# STANDARD OPERATING PROCEDURE

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<th>Title:</th>
<th>Immunofluorescence (IF) Microscopy using EVOS™ M5000 Imaging System</th>
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<td>M-137</td>
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## 1. PURPOSE

This procedure will describe how to prepare antibodies/protein targets for immunofluorescence (IF). The procedure utilizes fluorescent-labeled antibodies to detect specific target proteins at the subcellular level, followed by imaging using the EVOS™ M5000 Imaging System to provide information such as protein expression and localization.

## 2. SCOPE

This procedure applies to all samples that are processed for immunofluorescence analysis using EVOS™ M5000 Imaging System. This procedure incorporates the Human Protein Atlas (HPA) IF staining protocol for adherent and suspension cells using a 96-well microplate format but uses the EVOS™ M5000 Imaging System. The fixation and permeabilization of cells, primary and secondary antibody incubations 4′,6-diamidino-2-phenylindole (DAPI) fluorescent nuclear staining and mounting procedures will be described in this SOP.

## 3. RESPONSIBILITIES

It is the responsibility of person(s) performing this procedure to be familiar with cell culture, paraformaldehyde (PFA)/lab safety procedures and the use of the EVOS™ M5000 imaging system. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation. It is the responsibility of the analyst to follow the procedure and document any deviations and all observations in a laboratory notebook.
4. **EQUIPMENT**

- Invitrogen™ EVOS™ M5000 Imaging System; Thermo Fisher Scientific
- Standard Laboratory Cell Culture CO₂ Incubator
- Biosafety Cabinet/Hood Class II
- Laboratory Chemical Fume Hood
- Centrifuge with swing rotor for microplates, Eppendorf, Model # 5810 R
- Vortex
- pH meter
- Stirring Hot plate

5. **MATERIALS**

Use the following recommended materials, or equivalent:

- Greiner Sensoplate™ glass bottom multi-well plates; Millipore Sigma, Cat. # M4187-16EA
- Shi-fix™ coated 96-well microplate; Everest Biotech, Cat. # SB-Shifix96
- 250 ml beaker and magnetic stir bars
- Concentrated NaOH
- Concentrated HCl
- Deionized (DI) Water (MilliQ or better)
- 10X Phosphate Buffered Saline (PBS); Fisher Scientific, Cat. #BP399-1 (diluted to 1X with DI water)
- Superfibronection; Millipore Sigma, Cat. # S5171-.5MG
- Heat Inactivated Fetal Bovine Serum (FBS), Life Technologies, Cat. # 16140071
- 16% Paraformaldehyde (PFA); VWR, Cat. # BT140770-10X10
- 4% PFA/1X PBS **Prepare as follows:**  
  
  _Always use lab coat, eye protection and gloves and perform work in a fume hood when preparing and using 4% PFA/1X PBS._  
  1. Pre-heat 75 ml 10% FBS diluted with1X PBS in a 250 ml beaker to 60°C on a hot plate with magnetic stirrer.  
  2. Add 25 ml 16% PFA to the warm FBS/PBS (Step 1) and continue stirring for 20 min at 60°C.  
  3. Slowly add concentrated NaOH to the solution from Step 2 until the solution reaches pH ~11.  
  4. Allow the solution to cool down to room temperature (~ 20°C).
5. Adjust pH to 7.2-7.3 using first concentrated HCl, and then diluted HCl for fine adjustment.
6. Aliquot solution and store at -20°C until use. Thaw at room temperature right before use.

- Triton X-100 Solution; Millipore Sigma, Cat. # 93443-100ML
- DAPI; VWR, Cat. # 10180-614. **Prepare DAPI solution as follows:**
  1. Dissolve 10 mg of DAPI powder in 2 ml MilliQ water to a 5 mg/ml (14.3 mM) concentrated stock solution. Store at -20°C until needed.
  2. When ready to use thaw 5 mg/ml DAPI stock solution at room temperature, then combine 80 μl of 5 mg/ml DAPI stock solution with 920 μl MilliQ water to a pre-diluted 400 μg/ml DAPI solution. Aliquot and store extra vials at -20°C until needed.
  3. When ready to use thaw pre-diluted 400 μg/ml DAPI solution at room temperature to prepare 0.2 μg/ml DAPI with 1X PBS (0.2 μg/ml DAPI/1X PBS) for experiment. Discard any unused 0.2 μg/ml DAPI/1X PBS.
- Aluminum PCR Plate Sealers, Greiner Bio-One; VWR, Cat. # 82050-998
- Pipettes and tips
- Micro centrifuge and tubes
- 50 ml 0.2 μm filter units; Thermo Fisher Scientific, Cat. # 564-0020
- Glycerol; Thermo Fisher Scientific, Cat. # 15514011
- Glycerol/10X PBS. **Prepare as follows:**
  1. Add 5 ml 10X PBS to 45 ml of glycerol. Mix by inverting.
  2. Autoclave or filter sterilize.
  3. Store at Room Temperature

6. REAGENTS

6.1. For Rabbit or Mouse Monoclonal Antibody Evaluation

- Cells corresponding to the antibody or protein of interest such as cell lines (suspension or adherent) or equivalent. Purified monoclonal antibody corresponding to the protein of interest
- Mouse or rabbit anti-alpha tubulin marker; Abcam, Cat. # Ab7291 or Ab176560, respectively.
- Goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 555 secondary antibodies; Thermo Fisher Scientific, Cat. # A11034 and A21424, respectively or other species as appropriate conjugated Alexa Fluor secondary antibodies.
7. PROCEDURE

7.1. Guidelines
- All volumes in the protocol refer to volume per well in a 96-well microplate. Unspecified incubation temperatures are at room temperature. Immunostaining can be performed manually or using a liquid handling robot.

7.2. Preparation of Primary Antibodies
- Before immunostaining, dilute primary antibodies to 2 ug/ml in Blocking Buffer (4% FBS/1X PBS) together with 1 ug/ml anti-alpha tubulin marker in one tube.

7.3. Preparation of Secondary Antibodies
- Before immunostaining, dilute secondary antibodies to 2.5 ug/ml in Blocking Buffer (4% FBS/1X PBS) and protect from light.

7.4. Cell Culture
- Grow adherent cells overnight in 80 µl cell culture media (according to cell provider’s instructions) on fibronectin (12.5 µg/ml) coated 96-well glass bottom microplate ( precoated according to manufacturer instructions). Note, optimal seeding concentration/confluence for IF staining varies between cell lines and test samples may be required to define optimal conditions for desired cell line/target. For suspension cells (10 million cells/mL in 1X PBS) use the Shi-fix™ coated 96-well microplate at a recommended concentration between 200,000 -500,000 cells/well. Follow Shi-fix™ manufacturer’s instructions and proceed to fixation. Note, optimal cell count for IF staining varies between cell lines and test samples may be required to define optimal conditions for desired cell line/target.

7.5. Fixation
- Remove cell culture media (aspirate ~ 80 µl from all wells).
- Wash cells once gently with 40 µl 1X PBS.
- Fix cells by incubating with 40 µl of 4% PFA/1X PBS for 15 minutes.

7.6. Permeabilization
- Remove the 4% PFA/1X PBS and permeabilize the cells by incubating with 40 µl 0.1% Triton X-100/1X PBS for 5 minutes and repeat 3X.
7.7. **Primary Antibody incubation**
- Add 40 µl Blocking Buffer (4% FBS/1X PBS) with diluted primary antibodies and anti-α tubulin marker (Step 7.2).
- Incubate overnight at 4°C or for two hours at room temperature.

7.8. **Secondary Antibody incubation**
- Remove Blocking Buffer containing primary antibodies and gently wash cells with 40 µl 1X PBS for 10 minutes repeat 4X.
- Add 40 µl Blocking Buffer containing diluted secondary antibodies (Step 7.3)
- Incubate at room temperature for 90 minutes in the dark.

7.9. **DAPI Nuclear Staining and Mounting**
- Remove Blocking Buffer containing secondary antibody and incubate with 40 µl 0.2 µg/ml DAPI/1X PBS for 10 minutes.
- Gently wash the cells with 40 µl 1X PBS for 10 minutes repeat 4X.
- Add glycerol/10X PBS to cover the cells (~200 ul) and seal the plate with an adhesive aluminum PCR plate seal.
- Image the cells immediately or store plates at 4°C for no more than two weeks before imaging for best results.

8. **REFERENCED DOCUMENTS**


8.2 Shi-fix™ coated 96-well microplate protocol, Everest Biotech Product Datasheet